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Early microglial inhibition preemptively mitigates chronic pain development after experimental spinal cord injury

Andrew M. Tan, PhD; Peng Zhao, PhD; Stephen G. Waxman, MD, PhD; Bryan C. Hains, PhD*
Rehabilitation Research Center, Department of Veterans Affairs Connecticut Healthcare System, West Haven, CT; Center for Neuroscience and Regeneration Research, Department of Neurology, Yale University School of Medicine, West Haven, CT

Abstract—Spinal cord injury (SCI) results in the development of chronic pain syndromes that can persist indefinitely and cause reductions in quality of life. Treatment of chronic pain after SCI remains extremely challenging; thus, an important research goal is to determine whether early treatments can attenuate the subsequent development of pain conditions. The current study examined the hypothesis that early administration of the microglial-inhibiting drug minocycline could ameliorate the development of pain after SCI. Adult male Sprague-Dawley rats underwent SCI at the ninth thoracic spinal segment and received either vehicle or minocycline treatment for 5 days postinjury. Time course studies revealed that over 4 weeks post-SCI, microglial activation in vehicle-treated animals was progressively increased. Minocycline treatment resulted in reduction, but not prevention, of microglial activation over time. Electrophysiological experiments showed that early minocycline administration attenuated the development of chronic hyperresponsiveness of lumbar dorsal horn neurons. Similarly, behavioral assessment showed that minocycline also resulted in increased pain thresholds. These results suggest that inhibition of early neuroimmune events can have a powerful impact on the development of long-term pain phenomena following SCI and support the conclusion that modulation of microglial signaling may provide a new therapeutic strategy for patients suffering from post-SCI pain.

Key words: contusion, dorsal horn, hypersensitivity, microglia, microglial-activation, minocycline, pain, rehabilitation, SCI, spinal cord injury.

INTRODUCTION

Multiple biochemical, neuronal, and glial mechanisms contribute to chronic pain induction and maintenance after spinal cord injury (SCI). Glial cells in particular are very responsive to injury and have been implicated in pain syndromes following SCI and nerve injury. Researchers have known for some time that neuroimmune cells called microglia become activated in the spinal cord after clinical [1] and experimental [2–13] SCI but only recently have the functional implications of microglial activation for pain processing after SCI been explored by our laboratory and others [7,9].

Abbreviations: BBB = Basso, Beattie, and Bresnahan (scale); CCL21 = chemokine (C-C motif) ligand 21; ERK = extracellular signal-regulated kinase; IL = interleukin; IP = intraperitoneal, L + number = lumbar spinal segment; MAP = mitogen-activated protein (kinase); PARP-1 = poly(ADP-ribose) polymerase-1; PB = phasic brush; PBS = phosphate-buffered saline; PGE2 = prostaglandin E2; SC = subcutaneous, SCI = spinal cord injury; T9 = ninth thoracic; TNF-α = tumor necrosis factor-alpha; VPL = ventral posterolateral.

***Address all correspondence to Bryan C. Hains, PhD; Center for Neuroscience and Regeneration Research, Department of Neurology, Yale University School of Medicine, 950 Campbell Avenue, Bldg 34, West Haven, CT 06516; 203-932-5711, ext 3663; fax: 203-937-3801. Email: bryan.hains@yale.edu**

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Our recent work has shown that thoracic SCI causes microglial cells to activate at multiple levels along the sensory neuraxis and to contribute to the maintenance, in real time, of hyperexcitability of dorsal horn and thalamic somatosensory neurons, which leads to chronic pain [10,12–13]. We have elucidated a direct microglia-neuron signaling mechanism that involves prostaglandin E2 (PGE2), regulated in microglia by extracellular signal-regulated kinase (ERK)1/2 mitogen-activated protein (MAP) kinase [12], which serves as a way by which activated microglia can enhance the excitability of pain-processing neurons. We have also worked out the mechanism by which the SCI triggers remote microglial activation in the thalamus via the chemokine (C-C motif) ligand 21 (CCL21) [13], which triggers a cascade that results in neuronal hyperexcitability [14–16].

While our data suggest a powerful role in pain modulation and signaling by activated microglia at chronic time points after injury, little is known about the time course of microglial activation after SCI. Similarly, while evidence suggests that early microglial inhibition can result in neuroprotection after SCI [17–18], little is known about the importance and functional consequences of early microglial activation or inhibition of microglial activation on pain processing at chronic time points. Thus, the purpose of the current set of experiments was twofold. We first characterized the time course of activation of lumbar dorsal horn microglia after SCI. Second, we determined whether inhibition of microglial activation near the time of injury could mitigate the development of chronic pain. Here we report that early microglial inhibition results in reductions in neuronal hyperexcitability and pain-related behaviors at more chronic time points following SCI.

METHODS

Animal Care

Experiments were carried out in accordance with National Institutes of Health guidelines for the care and use of laboratory animals; all animal protocols were approved by the Yale University Institutional Animal Use Committee. Adult male Sprague-Dawley rats (200–225 g, Harlan Laboratories; Indianapolis, Indiana) were used for this study. Animals were housed under a 12 h light-dark cycle in a pathogen-free area with free access to water and food.

Spinal Cord Contusion

Rats were deeply anesthetized with intraperitoneal (IP) injection of ketamine/xylazine (80/5 mg/kg). Spinal cord contusion was produced (n = 34 rats) at the ninth thoracic (T9) spinal segment using the MASCIS/NYU (Multicenter Animal Spinal Cord Injury Study/New York University) impact injury device [19]. A 10 g, 2.0 mm-diameter rod was released from a 25 mm height onto the exposed spinal cord. For sham surgery, animals (intact, n = 8) underwent laminectomy and placement into the vertebral clips of the impact injury device without impact injury. After SCI or sham surgery, the overlying muscles and skin were closed in layers with 4-0 silk sutures and staples, respectively, and the animals were allowed to recover on a 30 °C heating pad. Postoperative treatments included saline (2.0 mL subcutaneous [SC]) for rehydration and Baytril (0.3 mL, 22.7 mg/mL SC, twice daily) to prevent urinary tract infection. Bladders were manually expressed twice daily until reflex bladder emptying returned, typically by 10 days postinjury. Following surgery, animals were maintained under the same preoperative conditions and fed ad libitum.

Drug Delivery

Drug injections began on the day of injury and lasted a total of 6 days (to day 5 after injury). The tetracycline antibiotic minocycline has been shown to potently downregulate the activity of microglia [20–22] and to inhibit inflammatory cytokines, free radical production, and matrix metalloproteinases [23]. We used 7-dimethylamino-6-demethyl-6-deoxytetracycline (minocycline hydrochloride, molecular weight 493.9; Sigma-Aldrich, Co; St. Louis, Missouri) as described in previous reports [20,24]. Five minutes after SCI, minocycline (90 mg/kg in 0.3 mL IP) was given to a subset of animals (n = 17); 0.9 percent saline vehicle was given to the remaining rats (0.3 mL IP, n = 17). For 5 additional days, minocycline (45 mg/kg in 0.3 mL IP, twice daily) or saline vehicle (0.3 mL IP, twice daily) was injected.

Immunohistochemistry

Tissue was collected from the lumbar enlargement (L4 spinal segment) of animals from the following groups: intact (n = 3), SCI + vehicle (n = 12), or SCI + minocycline (n = 12). Rats were deeply anesthetized with ketamine/xylazine (80/5 mg/kg IP) and perfused intracardially with 0.01M phosphate-buffered saline (PBS) followed by 4 percent cold buffered paraformaldehyde. Tissue collection was performed weekly for 4 weeks beginning 1 week following

SCI. Tissue was postfixed for 15 min in 4 percent paraformaldehyde and cryopreserved overnight at 4 °C in 30 percent sucrose PBS. Thin cryosections (8 µm) from each treatment group (n = 6 sections/animal) were processed simultaneously using a procedure that permits visualization of microglia [10]. Slides were incubated at room temperature in (1) blocking solution (PBS containing 5% normal goat serum, 2% bovine serum albumin, 0.1% Triton X-100, and 0.02% sodium azide [Sigma-Aldrich, Co]) for 30 min; (2) mouse anti-CD11b/c OX-42 clone raised against complement receptor 3 (1:250, BD Biosciences; San Jose, California) overnight in blocking solution at 4 °C; (3) PBS, 6× for 5 min each; (4) goat anti-mouse Alexa 546 (1:1500, Molecular Probes; Eugene, Oregon) in blocking solution for 2 h; and (5) PBS, 6× for 5 min each. Control experiments were performed without primary or secondary antibodies, yielding only background levels of signal.

Quantitative Image Analysis

Images were captured with a Nikon 80i light microscope (Nikon; Tokyo, Japan) equipped with epifluorescence optics and a Retiga EXi camera (QImaging; Surrey, British Columbia, Canada). Quantification of digitally captured images (n = 4–7 sections/3 animals/group) was performed by a blinded observer using Nikon Elements AR 2.30 software. Percent of field analysis was used to provide a quantitative estimate (proportional area) of changes in the activation state of microglial cells [2,10,12–13] in dorsal horn laminae I to IV based on atlas boundaries, after subtraction of background signal. Sensing/quiescent ("resting") microglia were classified as having small compact somata bearing long thin ramified processes. Activated microglia exhibited marked cellular hypertrophy and retraction of processes such that the process length was less than the somal diameter. Background levels of signal were subtracted and control and experimental conditions evaluated in identical manners.

Electrophysiological Procedures

Extracellular unit recordings were obtained from dorsal horn sensory neurons 30 days after SCI. The activity of 5 to 6 units/animal (n = 3/group from intact, SCI + vehicle, and SCI + minocycline) was recorded for each experiment, yielding 15 to 18 cells/group. Rats were initially anesthetized with sodium pentobarbital (40 mg/kg IP) and supplemented (5 mg/kg/h) as needed. The spinal cord was exposed by lumbar laminectomy before fixation of the head and the vertebral column on a stereotaxic apparatus. The exposed spinal cord was covered with warm (37 °C) mineral oil. Units were isolated from lumbar (L)3 to L5 medially near the dorsal root entry zone up to a depth of 1,000 µm. Recordings were obtained with a low-impedance 5M tungsten-insulated microelectrode (A-M Systems; Carlsborg, Washington). Electrical signals were amplified and filtered at 300 to 3,000 Hz (DAM80, World Precision Instruments; Sarasota, Florida), processed by a data collection system (CED 1401+, Cambridge Instruments; Cambridge, United Kingdom), and stored on a computer (Latitude D800, Dell; Austin, Texas). The stored digital record of individual unit activity was retrieved and analyzed offline with Spike2 software (v5.03, Cambridge Electronic Design; Cambridge, United Kingdom).

After a cell was identified and its receptive field was mapped, natural stimuli were applied: (1) phasic brush (PB) stimulation of the skin with a cotton applicator; (2) compressive pressure, by attaching a large arterial clip with a weak grip to a fold of skin (144 g/mm^2); (3) compressive pinch, by applying a small arterial clip with a strong grip to a fold of skin (583 g/mm^2); followed by (4) graded intensity-calibrated von Frey filaments (0.39, 1.01, and 20.8 g) (Stoelting; Wood Dale, Illinois). Multireceptive neurons were identified by their relative magnitude of responsiveness to all stimuli. Stimulation was applied with the experimenter blinded to the output of the cell during stimulation. Background activity was recorded for 10 s, and stimuli were applied serially for 10 s, separated by another 10 s of spontaneous activity without stimulation. Care was taken to ensure that the responses were maximal, that each stimulus was applied to the primary receptive field of the cell, and that isolated units remained intact for the duration of each experiment by using Spike2 template-matching routines. On the basis of previously published statistical analysis of evoked discharge rates in intact control and SCI animals [5,25–27], we considered neurons to be hyperresponsive if evoked discharge rates were >150 percent of control levels.

Behavioral Testing

Behavioral testing was performed by a blinded observer (n = 5 animals/group). Testing began on day 28 after SCI to confirm that animals had recovered enough motor function to enable testing of nociceptive thresholds [28]. Locomotor function was recorded using the Basso, Beattie, and Bresnahan (BBB) rating scale [29] to ensure reliability of hindlimb somatosensory testing. The BBB is a 21-point ordinal scale ranging from 0, which is no discernable hindlimb movement, to 21, which is consistent and coordinated gait with parallel paw placement of the hindlimb and consistent trunk stability. Scores from 0 to 7 rank

the early phase of recovery with return of isolated movements of three joints (hip, knee, ankle); scores from 8 to 13 describe the intermediate recovery phase with return of paw placement, stepping, and forelimb-hindlimb coordination; and scores from 14 to 21 rank the late phase of recovery with return of toe clearance during the step phase, predominant paw position, trunk stability, and tail position. We tested nociceptive thresholds in animals with BBB scores above 9.

Mechanical nociceptive thresholds were determined by assessing paw withdrawal to application of a series of calibrated von Frey filaments to the glabrous surface of the hindpaws. Before testing, animals were acclimatized to the testing area for 30 min. Following application of von Frey filaments (0.4–26 g) with enough force to cause buckling of the filament, we used a modification of the "up-down" method of Dixon [30] to determine the value at which paw withdrawal occurred 50 percent of the time [31], interpreted to be the mechanical nociceptive threshold.

After the animals acclimatized to the test chamber, thermal nociceptive thresholds were assessed by measuring the latency of paw withdrawal in response to a radiant heat source [32]. Animals were placed in Plexiglas boxes on an elevated glass plate (37 °C) under which a radiant heat source (5.14 amps) was applied to the glabrous surface of the paw through the glass plate. The heat source was turned off automatically by a photocell upon limb-lift, allowing the measurement of paw-withdrawal latency. If no response was detected, the heat source was automatically turned off at 20 s. Three minutes were allowed between each trial, and four trials were averaged for each limb.

Statistical Analysis

Data were evaluated at a level of 0.05 by two-tailed analyses. Within-group measures were tested for significance by using one-way analysis of variance and Tukey's post-hoc test. Data management and statistical analyses were performed using SAS statistical procedures (SAS Institute, Inc; Cary, North Carolina) and graphed using SigmaPlot as mean ± standard deviation (version 7.0; Systat Software, Inc; Chicago, Illinois).

RESULTS

Immunohistochemistry

Cd11b/c immunostaining revealed the presence of microglia in the lumbar dorsal spinal cord of all animals examined (**Figure 1**). In intact animals (**Figure 1(a)**), the dominant morphology was resting, whereby microglia displayed small compact somata bearing long thin ramified processes (**Figure 1(j)**). After SCI, all groups demonstrated increased microglial activation, which continued throughout the duration of the experiment (4 weeks, **Figure 1(b)–(i)**). Activated microglia exhibited marked cellular hypertrophy and retraction of processes (**Figure 1(k)**). A lesser degree of microglial activation was apparent from examination of tissue from SCI animals treated with early minocycline when compared with vehicle-treated animals. Quantification of Cd11b/c field area as an indicator of microglial activation revealed significant differences between vehicle and minocycline groups starting at 2 weeks post-SCI ($p < 0.05$) (**Figure 2**). By 4 weeks after injury, animals that received minocycline (157.6 ± 12.2 arbitrary units) demonstrated reductions in activation of up to 28 percent compared with those that received the vehicle (216.6 ± 24.7 arbitrary units) ($p = 0.001$).

Extracellular Unit Recordings

Dorsal horn multireceptive units were sampled in the lumbar enlargement (L3–L5) in intact animals and after SCI with or without early minocycline administration. **Figure 3** shows the effects of early minocycline administration on peripherally evoked activity 30 days after SCI. A representative unit from a sham animal displaying evoked responses is shown in **Figure 3(a)** for comparison. In comparison to sham animals, SCI animals displayed increased peripherally evoked unit responses for all stimuli (**Figure 3(b)**): for PB, 7.4 ± 4.4 Hz versus 43.0 ± 15.3 Hz; for pressure, 6.5 ± 5.0 Hz versus 32.4 ± 9.2 Hz; for pinch, 7.9 ± 5.3 Hz versus 38.8 ± 22.9 Hz; for 0.39 g von Frey filament, 4.8 ± 4.7 Hz versus 16.5 ± 5.8 Hz; for 1.01 g von Frey filament, 6.5 ± 5.7 Hz versus 22.8 ± 9.1 Hz; and for 20.8 g von Frey filament, 8.6 ± 7.1 Hz versus 31.5 ± 15.4 Hz ($p < 0.001$ for all). PB stimulation as well as compressive press and pinch stimuli resulted in high-frequency discharge. Von Frey filament stimulation resulted in graded increases in responsiveness of sampled units ($p < 0.001$) (**Figure 3(d)**). Minocycline administration resulted in decreased evoked responses to all peripheral stimuli (**Figure 3(c)**). In SCI animals, early minocycline treatment significantly reduced the evoked responses to all peripheral stimuli recorded 30 days after SCI ($p < 0.001$) (**Figure 3(d)**): for PB, 9.9 ± 6.1 Hz versus 43.0 ± 15.3 Hz; for pressure, 9.6 ± 5.0 Hz versus 32.4 ± 9.2 Hz; for pinch, 11.8 ± 5.3 Hz versus 38.8 ± 22.9 Hz; for 0.39 g von Frey filament, 4.3 ± 2.6 Hz versus 16.5 ± 5.8 Hz; for 1.01 g von Frey filament, 5.6 ± 3.3 Hz versus

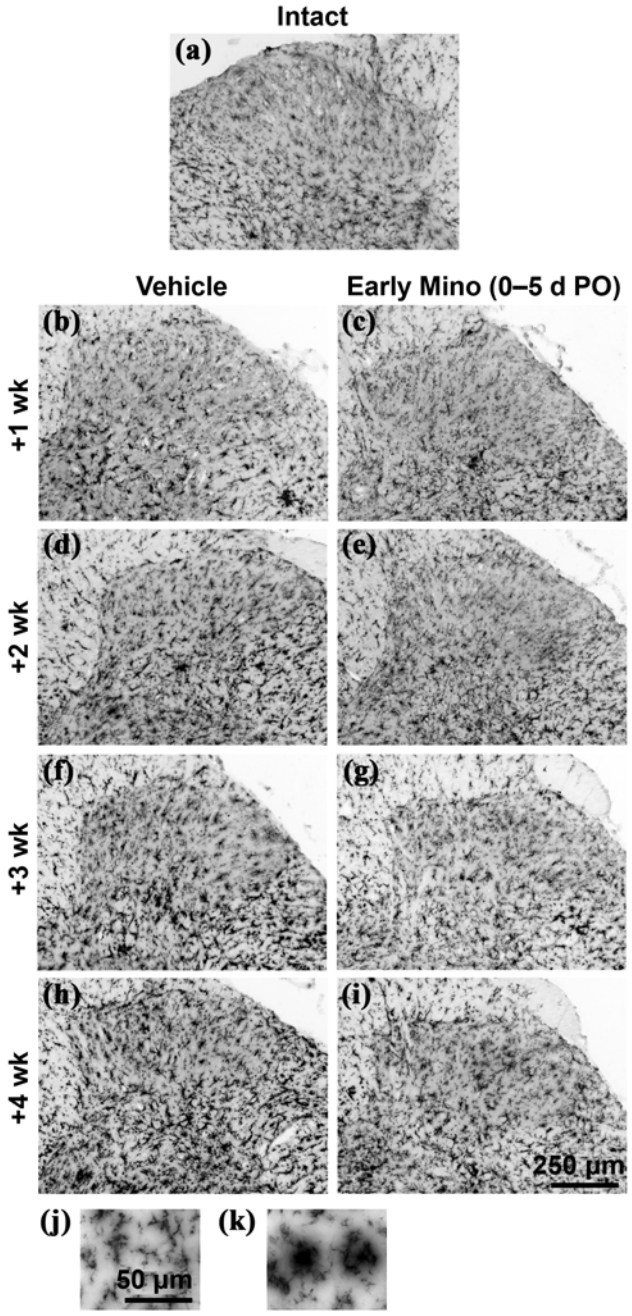

Figure 1.
Microglial activation after spinal cord injury (SCI). **(a),(j)** In intact animals, microglial activation status is predominately resting (small cell bodies extending thin ramified processes in all directions) in lumbar dorsal horn. **(b)–(i),(k)** After SCI, microglia undergo shifts in morphology to activated state whereby somas are more compact and processes are retracted. **(b),(d),(f),(h)** In animals treated with vehicle early after SCI, microglial activation occurred progressively over 4 weeks after injury. **(c),(e),(g),(i)** In animals treated acutely with microglial inhibitor minocycline (Mino), microglial activation was qualitatively reduced compared with vehicle-treated group. PO = postoperative.

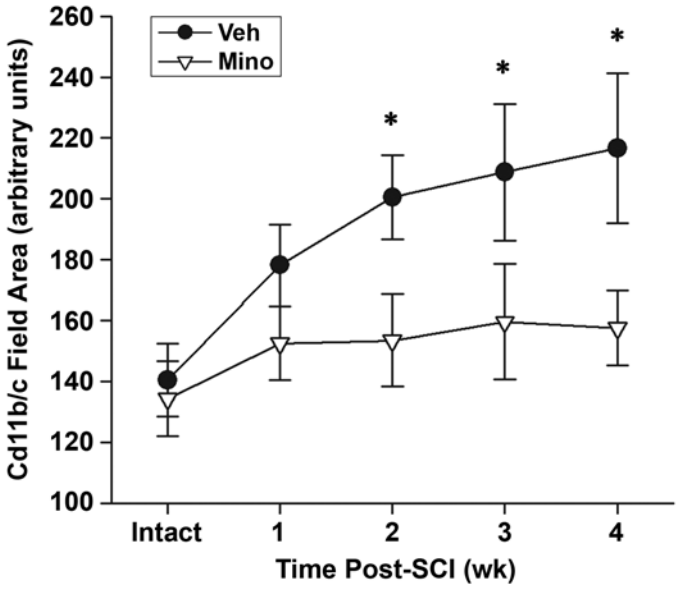

Figure 2.
Quantification of microglial activation. Analysis of Cd11b/c field area, indicator of microglial activation status, revealed that after spinal cord injury (SCI), microglial activation was progressive and increased over course of several weeks after SCI. Early administration of minocycline (Mino) (open triangles), but not vehicle (Veh) (filled circles), resulted in a significant reduction in microglial activation within lumbar dorsal horn, which was evident beginning at 2 weeks postinjury. $^{*}p < 0.05$.

22.8 ± 9.1 Hz; and for 20.8 g von Frey filament, 12.0 ± 4.6 Hz versus 31.5 ± 15.4 Hz ($p < 0.001$ for all). No significant differences were observed between sham animals and SCI animals treated early with minocycline.

Behavioral Testing

Before surgery, all animals demonstrated expected levels of locomotor function (group mean 21.0 ± 0.0), mechanical nociceptive threshold (group mean 20.4 ± 1.9 g), and thermal paw-withdrawal latency (group mean 9.9 ± 0.8 s). All drugs were given on the day of surgery and for 5 consecutive days following surgery, ending on postoperative day 6. For testing of the behavioral effects of vehicle or minocycline after SCI, we tested locomotor function on day 28 with the BBB locomotor rating scale and evaluated changes in mechanical and thermal nociceptive thresholds with standard thresholding paradigms.

Four weeks after SCI, BBB scores in sham-operated intact animals were unchanged (21.0 ± 0.0) (**Figure 4(a)**). At this same time point, SCI animals demonstrated significantly reduced BBB scores (9–12) compared with intact

animals. In animals that received vehicle injections early after SCI, BBB scores were 9.6 ± 0.5. In animals that received minocycline, BBB scores (11.2 ± 0.8) were significantly increased (p = 0.002) compared with vehicle-treated animals. These results indicate that minocycline treatment resulted in improved locomotor performance following SCI. We should note that the difference in BBB scores between treatment groups would not be expected to confer enhanced or decreased ability to respond to the stimuli used to test nociceptive thresholds.

After SCI, mechanical paw-withdrawal thresholds had significantly decreased in all groups (group mean 6.5 ± 4.1 g) compared with intact animals (20.1 ± 1.3 g) at day 28 (**Figure 4(b)**). Mechanical nociceptive thresholds were significantly increased (p = 0.003) in animals that received early administration of minocycline (9.7 ± 3.4 g) compared with vehicle (3.4 ± 1.4 g). This increase in paw-withdrawal threshold in the SCI + minocycline group is consistent with a reduced degree of mechanical allodynia.

Thermal paw-withdrawal latencies for all groups were significantly lowered after SCI, with a group mean of 6.6 ± 1.3 s (**Figure 4(c)**). In animals that received vehicle injections after injury, thermal paw-withdrawal latencies were 5.6 ± 1.0 s at day 28. In contrast, in animals that received minocycline, paw-withdrawal latencies

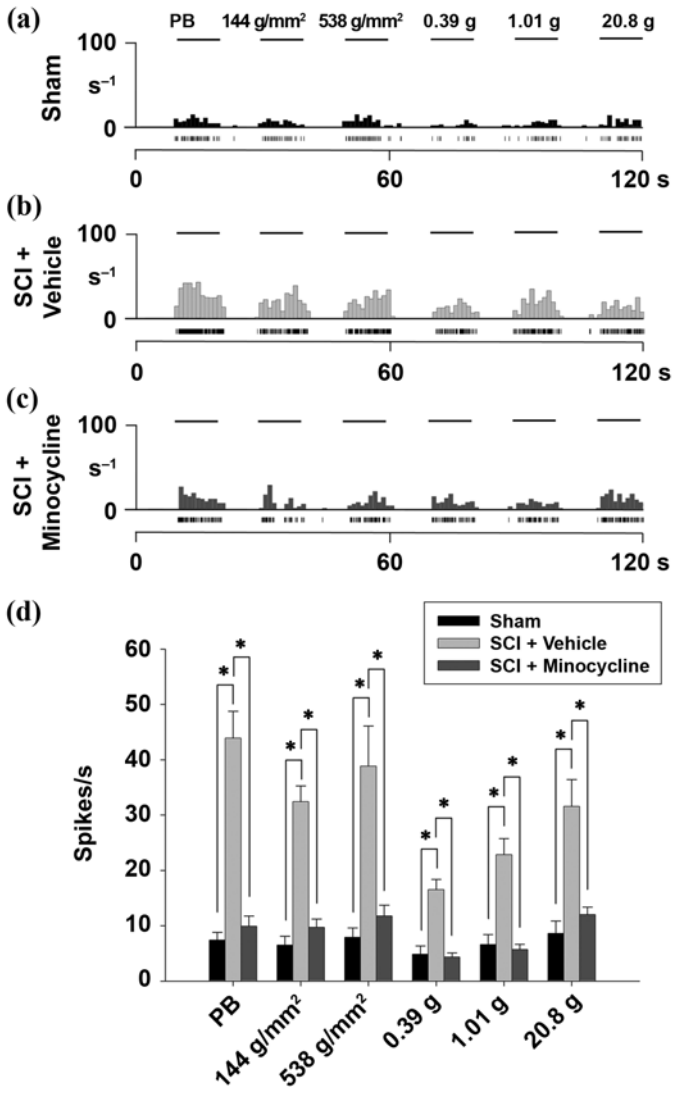

Figure 3.
Dorsal horn unit recordings. Early administration of minocycline attenuated peripherally evoked activity in multireceptive units from animals with spinal cord injury (SCI) 30 days after injury. **(a)** Representative unit from sham animal displayed evoked responses to stimuli (144 g/mm^2, 538 g/mm^2, 0.39 g, 1.01 g, and 20.8 g refer to pressure, pinch, and increasing intensities of von Frey filaments). **(b)** After SCI, animals showed increased evoked responses to all peripheral stimuli. **(c)** Peripherally evoked responses of all sampled multireceptive units decreased significantly after early minocycline treatment. **(d)** Compared with sham animals, SCI animals receiving vehicle demonstrated significantly increased evoked responses. Minocycline significantly reduced SCI-induced increases in responsiveness. $^*p < 0.05$. PB = phasic brush.

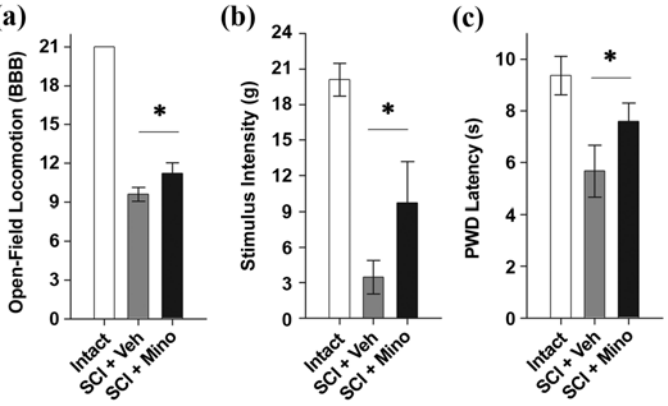

Figure 4.
Analysis of motor function and nociceptive thresholds. **(a)** At 4 weeks after injury, open-field locomotor scores were significantly reduced after spinal cord injury (SCI) when compared with intact animals; however, early administration of minocycline (Mino) resulted in significantly ($^*p < 0.05$) improved Basso, Beattie, and Bresnahan (BBB) scores compared with vehicle (Veh). **(b)** Mechanical nociceptive thresholds (stimulus intensity) were significantly reduced in all SCI animals in comparison to intact animals. In animals treated with Mino, however, hindpaw nociceptive thresholds were elevated in comparison to animals that received Veh. **(c)** Thermal paw-withdrawal (PWD) latencies showed significant reductions in both groups after SCI, but early administration of Mino resulted in increased PWD latencies.

were 7.5 ± 0.7 s, significantly different from vehicle treatment (p = 0.009), indicating a reduction in thermal hyperalgesia.

DISCUSSION

This study confirms that traumatic SCI results in the robust activation of microglia at spinal cord locations distant from the level of injury. In the present study, we report that microglial activation in the lumbar spinal cord dorsal horn follows an increasing temporal profile of activation after T9 contusion. We also show that early administration of the microglial-inhibiting agent minocycline can reduce the degree of neuronal hyperexcitability that develops in lumbar dorsal horn multireceptive neurons, as well as the degree of mechanical allodynia and thermal hyperalgesia at the 4-week time point after SCI. Our results reiterate the importance of microglia in nociceptive signaling after SCI and now show that early events triggered at the time of SCI play an important role in reconfiguring spinal nociceptive processing in such a way as to enhance the perception of pain after injury.

We have previously identified a putative signaling mechanism between activated microglia and pain processing neurons whereby PGE2 is a central molecule in microglia-mediated chronic pain [12]. pERK1/2 MAP kinase-mediated release of PGE2 from microglia, which binds to the EP2 receptor located on dorsal horn neurons, is sufficient to induce changes in the excitability state of these neurons and poises them to inappropriately amplify both innocuous and noxious sensory stimuli. Microglia may also release a number of additional proinflammatory cytokines and neuromodulators that contribute to hyperexcitability, such as p38 MAP kinase [9–10,21,33–35], interleukin (IL)-1 [36], IL-6 [37–38], and tumor necrosis factor-alpha (TNF-α) [22,39]. These circuit-level changes contribute to the expression of abnormal pain-related behaviors at a systems level.

The trigger for remote microglial activation after SCI at multiple locations along the sensory neuraxis, including the lumbar dorsal horn and thalamus, has recently been elucidated [13]. We demonstrated a mechanism whereby remote microglial activation is triggered by CCL21. CCL21 potently activates resident microglia within the ventral posterolateral (VPL) nucleus of the thalamus as well as the lumbar dorsal horn, and in turn, microglia pathologically modulate the firing properties of nociceptive neurons at these locations.

At chronic time points, pharmacological manipulations of neuron-microglia and microglia-neuron signaling either through inhibition of microglial activation, inhibition of ERK1/2 phosphorylation, antagonism of PGE2 receptor binding, selective killing of microglia with Mac-1-SAP, and/or inhibition of CCL21 signaling all result in reduced pain phenotypes [10,12–13]. However, in all of the conditions that have been studied, following wash out of compounds, pain phenomenology returned to predrug levels. Thus, some durability and persistence of microglia-mediated chronic pain after SCI exists, suggesting that a driving force maintains pain at time points and locations remote from the time and location of injury.

Early microglial inhibition may be a starting point from which preventative strategies could supplant the need for curative therapies later on. Thus, the driving force for microglial activation and signaling at later time points may be configured and cemented early after injury. Consistent with this hypothesis, inhibition of microglial activation at early time points (postoperative days 1–6) as shown here prevented the full-blown development of comparable neuronal hyperexcitability and reductions in nociceptive thresholds.

We cannot exclude the influence of higher-order nociceptive structures (i.e., the VPL and brain stem) in pain processing after SCI or drug treatment, which may play an important role in the enhancement or suppression of noxious stimuli from the spinal cord. These components may serve to modify responses and lead to differences in spinal cord unit recordings and behavioral output.

In the current experiments, we did not examine the effects of microglia-modulating agents at a chronic time point in early treated animals, but knowing how significant a role activated microglia play at chronic time points in animals that have received minocycline (or other microglial-modulating agents) at the time of injury will be important. If a reduced degree of chronic microglial activation/signaling exists in these animals, then we predict that lower doses of minocycline or other agents would be needed to obtain pain relief. In this case, early treatment might be expected to provide better prognosis than chronic treatment, since pain is more resilient in the latter case.

Given soon after contusion SCI, minocycline has been shown to improve BBB scores, reduce lesion volume, reduce signs of apoptosis, and reduce levels of TNF-α expression [17]. Another group showed similar results

whereby early minocycline treatment after SCI conferred neuroprotection, reduced microgliosis, and inhibited caspase-3 and TNF-α expression [18]. Although the exact mechanism underlying the effects of early minocycline treatment in our experiments is not known, we discuss several possibilities.

Tissue sparing at the site of SCI could possibly have the secondary effect of providing a descending pain inhibitory system with greater integrity as a result of neuroprotection; more specifically, the sparing of descending fibers such as the serotonergic medullary raphespinal tract that travels in the dorsolateral funiculus [40] and/or the catecholaminergic coerulospinal pathway originating in A5 and other nuclei could have this effect [41]. These fiber tracts are typically damaged or transected by contusion SCI [42]. Additionally, minocycline is known to inhibit poly(ADP-ribose) polymerase-1 (PARP-1), which promotes both cell death and inflammation, so that the neuroprotective and anti-inflammatory effects of minocycline and other tetracycline derivatives may be attributable to PARP-1 inhibition in some settings [43]. Finally, minocycline inhibits matrix metalloproteinases 2 and 9, which are involved with the degradation of the basal lamina and loss of stability of the blood-brain barrier that occur in ischemia [44–45]. The sum of these alterations may be increased survival of pain-modulatory systems. Protection of these systems may contribute toward the early minocycline effects of preventing chronic hyperexcitability of multireceptive neurons and expression of pain-related behaviors.

A mounting body of evidence demonstrates that minocycline treatment has a wide range of effects following SCI, which may be due to a number of factors (including a block in microglial activation). Our results clearly show a reduced level of microglial activation over the experimental time course in early minocycline-treated animals compared with vehicle-treated animals. In addition, 4 weeks after SCI, peripherally evoked activity in multireceptive units and pain-related behaviors in early minocycline-treated animals continued to remain significantly lower than in control animals. Together, these results highlight microglial activation as an important component of the pathophysiology in our model of SCI. Others have also shown similar effects on multireceptive dorsal horn neuronal excitability and pain behaviors [46]. Further work is necessary to identify the exact role of microglia in the early-stages of chronic pathological pain development.

Different populations of microglia likely play different roles at different locations at different times in the spinal cord after injury. Microglia may also play different roles in a variety of structures as well, which in the case of thalamic nociceptive neurons may include further amplification of signals received from spinal structures [13]. Watanabe et al. showed that microglia display at least two different spatial and temporal patterns of activation [47]. One is rapid and most likely involves the blood-borne complement-activating system. The other accompanies Wallerian degeneration and is independent of the blood-borne complement system. We cannot be sure which wave was affected in our model, but the most conservative conclusion is that both were affected by minocycline. With chronic administration, minocycline may also inhibit a unique pain-modulating population of microglia not involved in injury-associated inflammation (since after 4 weeks, the injury has mostly stabilized). Further work should investigate the sources of dorsal horn microglia and differences in microglial phenotype at different times and locations after SCI.

CONCLUSIONS

In summary, the current results and work by others establish inhibition of microglial activation as an important candidate for further study in terms of neuroprotection and pain relief following experimental SCI. At this time, minocycline is being evaluated as a candidate therapy to reduce microglial activation and its consequences in a number of neurological conditions [48], including Parkinson disease [49], Huntington disease [50–51], amyotrophic lateral sclerosis [52], multiple sclerosis [53–54], and stroke [55]. While the results of these studies have been mixed, the observations described here underscore the need for further investigation of microglial activation after SCI and of microglial-modulating agents as a potential therapeutic approach for chronic pain after SCI.

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The authors have declared that no competing interests exist.

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